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Molecular Genetics of *RECQL4* Syndromes

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Helsinki Graduate School in Biotechnology and Molecular Biology

Helsinki, Finland 2008

Annika Siitonen

**MOLECULAR GENETICS OF *RECQL4*
SYNDROMES**

ACADEMIC DISSERTATION

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University of Helsinki, for public examination in Lecture Hall 3,
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National Public Health Institute, Helsinki, Finland

and

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“Naisella on oltava rahaa ja oma huone, jos hän aikoo kirjoittaa.”

-Virginia Woolf

ABSTRACT

RAPADILINO syndrome is an autosomally recessively inherited condition that belongs to a group of rare syndromes more common in Finland than in other parts of the world. These syndromes are not only characterized by their prevalence in Finland, but also by the presence of a common founder mutation. RAPADILINO is characterized by pre- and postnatal growth retardation, radial ray defects, diarrhoea of unknown aetiology during childhood, a facial resemblance with other patients and normal intelligence. In Finland, 15 patients with this condition have been found which compares with only five patients in other parts of the world.

When mutations in the *RECQL4* gene were found in RAPADILINO patients, RAPADILINO syndrome was proven to be allelic with a subgroup of Rothmund-Thomson syndrome (RTS). Later on mutations in patients with Baller-Gerold syndrome (BGS) were also found in *RECQL4*. These syndromes are also characterized by pre- and postnatal growth retardation, radial ray defects and gastrointestinal problems. Differential diagnostics rely on poikiloderma, which is a hallmark feature of RTS found also in BGS patients, but not in RAPADILINO syndrome. Craniosynostosis is a feature found in BGS and the narrow definition of BGS is craniosynostosis with radial defects. RTS patients with *RECQL4* mutations have an elevated risk for osteosarcoma, but their risk to develop other types of malignancies does not increase. Two Finnish RAPADILINO patients have been diagnosed with osteosarcoma. In addition to this we have found an excess of lymphoma cases among the Finnish RAPADILINO patients.

The genotype-phenotype correlation is not straightforward but it seems that RAPADILINO could be due to alteration in protein function and truncating mutations in both alleles are more common among RTS patients. It has been shown that truncating mutations are linked to the osteosarcoma risk seen in RTS. We found five different mutations in the Finnish RAPADILINO patients. The g.2545delT mutation is the founder mutation in the Finnish population as all the patients are either homozygotes or compound heterozygotes for it. This mutation leads to the inframe skipping of exon seven from mRNA. The protein encoded by this mutant

mRNA lacks the nuclear retention signal and thus leads to the mislocalization of the mutant protein.

RECQL4 belongs to the RecQ gene family and there are five different RecQ genes in humans. Three of them - RECQL4, BLM and WRN - are linked to human syndromes which are characterized by growth retardation, dermatological changes and an elevated cancer incidence. Even though the exact role of RECQL4 is unknown it is proposed to take part in the maintenance of genomic integrity even if it lacks the helical activity that is present in all other RecQ proteins.

Keywords: RAPADILINO syndrome, Rothmund-Thomson syndrome, Baller-Gerold syndrome, *RECQL4*, RecQ gene family, osteosarcoma, lymphoma

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TIIVISTELMÄ

RAPADILINO-oireyhtymä on harvinainen suomalaiseen tautiperintöön kuuluva autosomaalisesti peittyvästi periytyvä oireyhtymä, joka on Suomessa diagnosoitu 15 henkilöllä ja muualla maailmassa viidellä potilaalla. Kuten muissakin suomalaisen tautiperinnön taudeissa Suomessa RAPADILINO-oireyhtymän taustalla on yksi yhteinen perustajamutaatio. Lisäksi on tunnistettu neljä muuta harvinaisempaa mutaatiota. RAPADILINO-oireyhtymä on nimetty pääoireidensa mukaan ja niiden esiintyvyys ja vakavuusaste vaihtelee potilaiden välillä. Useimmiten potilailla on jo ennen syntymää alkanut kasvuhäiriö, joka jatkuu syntymän jälkeen ja johtaa pienikasvuisuuteen. Lisäksi heillä on luuston kehityshäiriöitä, kuten puuttuvat tai alikehittyneet peukalot, varttinäluut sekä polvilumpiot. Potilaat kärsivät lapsuudessaan selittämättömästä syystä johtuvasta ripulista, joka yleensä paranee itsestään lasten vanhentuessa. Potilailla ei ole henkistä jälkeenjääneisyyttä ja he muistuttavat toisiaan enemmän kuin terveitä sisarusiaan.

RAPADILINO-oireyhtymän taustalta löytyivät mutaatiot *RECQL4*-geenissä, jonka mutaatioiden tiedettiin aiheuttavan osan Rothmund-Thomson –oireyhtymän tapauksista. Myöhemmin löysimme saman geenin mutaatiot myös Baller-Gerold –oireyhtymää sairastavilta potilaita. Nämä oireyhtymät ovat oireistoltaan osittain samanlaisia, sillä kaikkiin kuuluu kasvuhäiriö, peukaloiden, varttinäluiden sekä polvilumpioiden puuttuminen tai alikehittyneisyys. Erottavana piireitä ovat poikilodermaksi kutsuttu tyypillinen ihottuma RTS- ja BGS-potilailla sekä kraniosynostasia eli kallonluiden liian aikainen sulkeutuminen BGS-potilailla. Niillä RTS-potilailla, joilla on mutaatiot *RECQL4*-geenissä, on kohonnut riski sairastua pahanlaatuiseen luusyöpään, osteosarkoomaan. Suomalaisista RAPADILINO-potilaista kaksi on sairastanut osteosarkooman, ja tutkimukssessamme olemme myös havainneet suomalaisilla potilailla kohonneen lymfoomariskin.

Ilmiasun ja *RECQL4*-mutaatioiden välillä ei ole täydellistä yhteyttä, mutta RAPADILINO-oireyhtymää sairastavilla on useimmiten ainakin toisena mutaationa sellainen muutos, joka ei aiheuta geenin lukukehityksen tuhoutumista tai ennenaikaista katkeamista. Tällaisia ennenaikaisia lopetuskodoneita tai lukukehityksen sekoittavia mutaatioita löytyy useimmiten RTS-potilaita ja niiden ja osteosarkooman välillä on havaittu yhteys. Suomalaisilla RAPADILINO-potilailla

g.2545delT mutaatio aiheuttaa mRNA:n silmikoitumishäirön, joka johtaa eksonin seitsemän koodaaman alueen puuttumiseen mRNA:sta, muttei kuitenkaan tuhoa lukukehystä. Näin syntynyt mutanttiproteiini päättyy solussa solulimaan tuman sijasta, sillä siitä puuttuu tarvittava tumassapitosignaali.

RECQL4-proteiinin toimintaa ei tunneta vielä kunnolla, mutta se ilmeisesti toimii genomisen vakauden ylläpitäjänä muiden RecQ-proteiinien tavoin. RECQL4 kuuluu RecQ-geeniperheeseen johon ihmisellä kuuluu viisi geeniä - *RECQL*, *BLM*, *WRN*, *RECQL4* ja *RECQL5*. Mutaatiot *RECQL4*-, *BLM*- ja *WRN*-geeneissä aiheuttavat perinnöllisiä oireyhtymiä, joita yhdistävät ihomuutokset, pienikasvuisuus sekä syöpäalttius.

Avansanat: RAPADILINO-oireyhtymä, Rothmund-Thomson -oireyhtymä, Baller-Gerold -oireyhtymä, *RECQL4*-geeni, RecQ-geeniperhe, osteosarkooma, lymfooma

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ABBREVIATIONS

ATP	Adenosine triphosphate
BGS	Baller-Gerold syndrome
BLM	Bloom syndrome
bp	base pair
cM	centiMorgan
DSB	Double strand breaks
DNA	Deoxyribonucleic acid
GTP	Guanosine triphosphate
HR	Homologous recombination
HRDC	Helicase-and-ribonuclease D/C-terminal
HU	Hydroxyurea
kb	kilobase pair
LD	Linkage disequilibrium
Mb	megabase pair
MIM	Mendelian inheritance in man
NHJ	Nonhomologous end-joining
OS	Osteosarcoma
PCR	Polymerase chain reaction
PMA	Phorbol myristic acetate
PML	Promyelotic leukemia
Rad51	Homolog of RecA of <i>E. coli</i>
RNA	Ribonucleic acid
RECQL	RecQ like
RECQL4	RecQ like 4
RECQL5 β	RecQ like 5 β

RQC	RecQ conserved region
RTS	Rothmund-Thomson syndrome
SCE	Sister chromatin exchange
SGA	Small for gestational age
SNP	Single nucleotide polymorphism
TDT-LRT	Transmission disequilibrium test - likelihood ratio test
UBR1	Ubiquitin-protein ligase E3 component N-recognin 1
UBR2	Ubiquitin-protein ligase E3 component N-recognin 2
WRN	Werner syndrome

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals:

- I** **Siitonen HA**, Kopra O, Kääriäinen H, Haravuori H, Winter RM, Säämänen AM, Peltonen L & Kestilä M. Molecular defect of RAPADILINO syndrome expands the phenotype spectrum of *RECQL* diseases, *Hum Mol Genet*, (2003) 12:2837-2844

- II** Kellermayer R, **Siitonen HA**, Hadzsiev K, Kestilä M & Kostolányi G. A patient with Rothmund-Thomson syndrome and all features of RAPADILINO, *Arch Dermatol*, (2005) 141:617-620

- III** Van Maldergem L, **Siitonen HA**, Jalkh N, Chouery E, De Roy M, Delague V, Muenke M, Jabs EW, Cai J, Wang LL, Plon SE, Fourneau C, Kestilä M, Gillerot Y, Mégarbané A & Verloes A. Revisiting the Craniosynostosis-Radial ray hypoplasia association: Baller-Gerold syndrome caused by mutations in the *RECQL4* gene, *J Med Genet*, (2006) 43:148-152

- IV** **Siitonen HA**, Sotkasiira J, Biervliet M, Benmansour A, Cormier-Daire V, Crandall B, Hannula-Jouppi K, Hennekam R, Herzog D, Keymolen K, Lipsanen-Nyman M, Miny P, Plon S, Riedl S, Sarkar A, Vargas FR, Verloes A, Wang LL, Kääriäinen H & Kestilä M. Mutation spectrum in *RECQL4* diseases, submitted

Some unpublished data is also included into this thesis.

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1 INTRODUCTION

RAPADILINO syndrome is a rare condition found mainly in Finland, but some patients are found also in other parts of the world. RAPADILINO syndrome is characterized by growth retardation ($>-2SD$) and bone malformations such as hypoplasia of the thumbs, radius and patellae. Other clinical findings include diarrhoea, typical facial features, joint dislocations and normal intelligence. RAPADILINO syndrome is caused by mutations in the *RECQL4* gene. Ten Finnish patients were found to be homozygotes for the g.2545delT mutation (Fin_{major}) and five others were compound heterozygotes for this and another mutation. The Finnish founder mutation leads to inframe skipping of exon 7 and the four other mutations in Finnish population were truncating mutations.

Mutations in the *RECQL4* gene cause not only RAPADILINO, but also Rothmund-Thomson (RTS) and Baller-Gerold (BGS) syndromes. These three *RECQL4* syndromes have overlapping clinical findings such as growth retardation and radial ray defects, but poikiloderma and craniosynostosis are the hallmarks of their differential diagnostics. Patients share similar symptoms, but the syndromes are classified as separate diseases because of their different clinical findings. Patients with Rothmund-Thomson and Baller-Gerold syndromes have not been reported in Finland. In total 48 *RECQL4* mutations have been found, most of them being truncating mutations such as insertions, deletions, premature stop codons and splice site mutations. In general the genotype has a strong tendency to predict the phenotype, but it is not conclusive and this makes a thorough understanding of the *RECQL4* spectrum phenotypes demanding, but also rewarding.

For a long time RAPADILINO syndrome was thought to be a rare syndrome, only present in the isolated Finnish population. Yet it became an interesting example of a syndrome in which specific genetic defects lead to a distinct phenotype. When RAPADILINO and RTS were shown to be allelic the difference in the osteosarcoma incidence between these two syndromes was striking. Osteosarcoma has been reported to be much more common among RTS patients than in RAPADILINO patients. In addition to two osteosarcoma cases four Finnish RAPADILINO patients were found to develop lymphomas not described in RTS patients. This finding emphasizes the unique role of *RECQL4* at the cellular level. *RECQL4* is known to have high expression in chondrocytes, and skeletal defects with the elevated osteosarcoma incidence made it clear that *RECQL4* plays an important role in the developing bone. Because RAPADILINO patients are prone to lymphoma it can be assumed that *RECQL4* would have important functions in haematological cells as well.

2 REVIEW OF THE LITERATURE

2.1 Finnish disease heritage

The Finnish disease heritage is a group of 36 inherited conditions that are more common in Finland than in any other parts of the world (findis.org) (Perheentupa, 1972). These are all rare monogenic syndromes and 32 of them are recessive, two dominant and two X-linked (Norio, 2003c). The reason for this unique set of diseases in Finland is pure chance and the population history of this isolated country. (Norio, 2003b). On the other hand some diseases for example phenylketouria and cystic fibrosis that are common elsewhere are virtually nonexistent in Finland (Guldberg, et al., 1995; Kere, et al., 1994).

The molecular background of the diseases of the Finnish disease heritage has been effectively studied since the 1980's and the genetic defects underpinning all of these syndromes except Peho syndrome (Salonen, et al., 1991) are now known. In all the cases one prevalent founder mutation has been found to have the main contribution to the disease. In most diseases more than 90% of the Finnish patients have the same Fin_{major} mutation (Norio, 2003c). However, in some diseases the frequency of the Fin_{major} mutation is only about 70-80% and several minor mutations have been identified in Finnish and foreign patients, see e.g. (Huopaniemi, et al., 1999; Kestilä, et al., 1998; Kure, et al., 1992; Nagamine, et al., 1997; Sankila, et al., 1992).

2.2 Positional cloning

Isolated populations have been proven to be very useful in a disease gene hunting, for a review see e.g. (de la Chapelle and Wright, 1998; Norio, 2003c; Peltonen, et al., 1999). If the clinical examination is done thoroughly and the diagnostic criterion is kept strict enough there is a great probability that there is a common genetic defect behind the phenotype when studying isolated populations. But however it is possible that a same phenotype can be caused mutations in different genes (Paloneva, et al., 2000; Paloneva, et al., 2002). Church records in Finland are well kept and since they cover a long time span genealogical studies can also reveal common ancestors among the patients and thus confirm the assumption of a shared genetic background (Norio, 2003a).

In order to identify a disease-causing gene one first needs to collect DNA samples from the patients, their siblings and parents and genotype them by using a genome wide scan. From the genotype data one gets potential linkage to a genomic region

where the genetic defect can be found. Now when the human genome project has been completed and the genetic data is available in databases the positional cloning of the gene has become significantly easier and faster than earlier (Lander, et al., 2001; Venter, et al., 2001). However, in many cases the workflow is not as smooth and straightforward as it should be in theory. One of the restricting factors may be low numbers of samples if there are only a few patients with no siblings or samples from the patients' families are unavailable.

Because of the naturally occurring genetic variations between individuals genotyping them is possible (Botstein, et al., 1980). Nowadays in whole genome studies one can use either microsatellite markers (Dubovsky, et al., 1995) or single nucleotide polymorphisms (SNPs) (Frazer, et al., 2007) to get data for the study. Microsatellite markers are repetitive sequences where the same nucleotide combination is repeated several times. Di, tri and tetra nucleotide repeats are the most common repeat types. The best microsatellite markers are those that have several different alleles in the population. Hundreds of microsatellite markers are used in genome wide studies to detect linkage (Dubovsky, et al., 1995). If linkage is found in the initial screening, the chromosomal area can be further studied with additional markers. SNPs are polymorphisms of a single nucleotide that usually have no phenotypic effect (www.hapmap.org/). The numbers of SNPs detectable in the human genome is approximately 10 million. However, a smaller set of SNPs (e.g. 500 000) is big enough for genome wide studies. Certain SNP combinations occur together in so called haplotype blocks and these blocks can be identified by using tag SNPs. The human genome contains approximately 300 000 to 600 000 tag SNPs (www.hapmap.org/).

A combination of alleles of adjacent markers forms a haplotype and the haplotype is transmitted from one generation to the next. However, the haplotype becomes shorter, because recombinations occur between homologous chromosomes in meiosis. Thus in principle the older the ancestral haplotype is the shorter it has become. However, there are differences in the recombination probabilities in different parts of genome and in so called recombination hot spots the recombinations occur more often than in chromosomes in general (Broman, et al., 1998). Thus if a recombination hot spot is located in the studied chromosomal area the haplotype becomes shorter rapidly.

This phenomenon is explained in Figure 1 where the shortening of the original white chromosomal area after a series of recombinations can be seen. The founder mutation that emerges into the ancestral chromosome has been marked with a star. The number of patients and the size of families have a significant consequence since the more chromosomes there are to study the more likely informative recombinations can be observed.

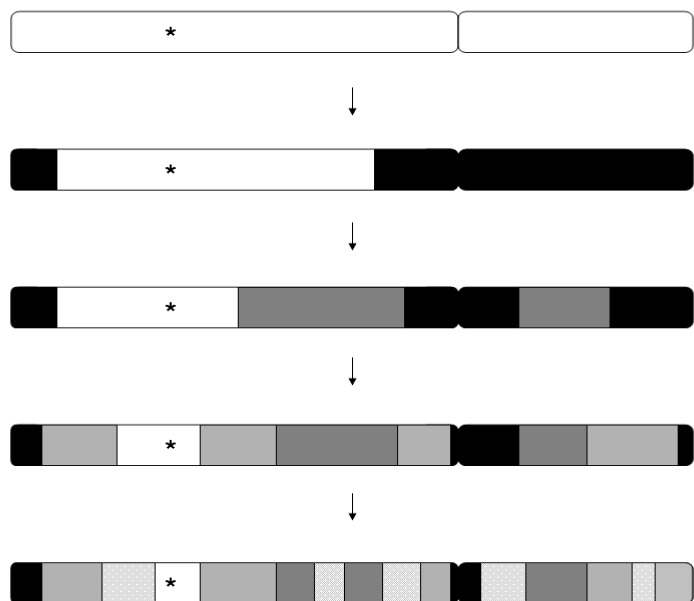


Figure 1. Possible outcome of recombinations in a founder chromosome after series of recombination.

When the genotype data from the families is available one can start to find shared regions between the patients. If patients share a common genomic area they seem to have certain alleles of the microsatellite markers more often than would be expected thus the genetic defect is linked to certain alleles of the markers. The closer the genetic defect and a marker are to each other the more likely they are to appear together from one generation to the next. This phenomenon is called linkage disequilibrium (LD) and usually getting linkage to a certain region over 1 cM in size by chance is low, but not impossible (Peterson, et al., 1995; Thompson and Neel, 1997). For example in monogenic diseases where a common founder mutation is expected one needs to find genomic areas where patients have the same haplotype. In dominant diseases the patients share the same haplotype in one of their chromosomes. In recessive disorders a mutation has to be present in both chromosomes. If patients are homozygotes for the mutation they usually share the same haplotype in their both chromosomes. In cases where patient is compound heterozygote for the founder mutation they have one chromosome that bears the common founder haplotype. Their second chromosome has a different haplotype since it is derived from a different ancestral chromosome.

In the diseases of the Finnish disease heritage the shared haplotype is usually long and the linkage disequilibrium can be detected over a several cM interval as for example in Northern epilepsy (Tahvanainen, et al., 1994). This of course makes the choice of candidate genes difficult, as there can still be tens of genes in the shared haplotype area. If the function of the genes are known or can be predicted the choice may be easy, but in some cases there is a tedious task of sequencing ahead.

2.3 RAPADILINO syndrome

The RAPADILINO syndrome (MIM 266280) is a rare autosomally recessively inherited disorder that is enriched in the Finnish population. The clinical findings in the first five patients prompted the clinicians to name this syndrome after the main manifestations which were RADial and PATellar hypo- or aplasia, high arched or cleft PALate, DISlocation of joints and DIarrhoea of unknown origin, LIMb malformations and LITtle size, long and slender NOse and NORmal intelligence (Kääriäinen, et al., 1989). The patients have normal hair, eyebrows and eyelashes and their only dermatological feature is brownish spots resembling irregularly shaped café-au-lait spots (Kääriäinen, et al., 1989).

After this initial report three cases have been described outside Finland (Jam, et al., 1999; Kant, et al., 1998; Vargas, et al., 1992). All these patients had classical features of RAPADILINO and in two cases the phenotypes were compared to other similar phenotypes for differential diagnostics (Kant, et al., 1998; Vargas, et al., 1992). RAPADILINO was thus assumed to be present also in other parts of the world and not to be a syndrome found only in Finland. Later, one non-Finnish patient was re-diagnosed to suffer from Rothmund-Thomson syndrome (Hilhorst-Hofstee, et al., 2000; Kant, et al., 1998). The clinical picture of seven RAPADILINO patients is shown in Table 1. Since strict criteria was used, patients have very similar phenotypes although rare findings such as malsegmentation of cervical spine and brownish spots on skin were also reported (Jam, et al., 1999; Kant, et al., 1998; Kääriäinen, et al., 1989).

Table 1. Clinical data from seven RAPADILINO patients

	1	2	3	4	5	6	7
Short stature	+	+	+	+	+	+	+
Hypoplastic or absent radius	+	+	+	+	+	+	+
Hypoplastic or absent thumb	+	+	+	+	+	+	+
Hypoplastic or absent patellae	+	+	+	+	+	+	+
Joint dislocation	-	+	+	-	+	-	+
Cleft or high arched palate	+	+	+	+	+	+	+
Diarrhoea	+	+	+	+	+	+	+
Normal intelligence	+	+	+	+	+	+	+
Typical face	+	+	+	+	+	+	+
High pitched voice	+	+	+	+	+	+	+
Brownish spots	+	-	-	-	-	*	-

Patients 1 to 5 are the first RAPADILINO patients described (Kääriäinen, et al., 1989). Patient 6 is the first non-Finnish patient (Vargas, et al., 1992) and patient 7 the second non-Finnish RAPADILINO case (Jam, et al., 1999).

** Patient has a dark skin color so brownish spots would not be visible.*

2.4 Rothmund-Thomson syndrome

Originally, the Rothmund-Thomson syndrome (RTS, MIM 268400) was actually two separate entities described by Rothmund (Rothmund, 1887) and Thomson (Thomson, 1936). Rothmund's eight patients were from three families from an isolated Bavarian alpine valley (Rothmund, 1887). Thannhauser (1945) gives an English translation of the clinical descriptions in Rothmund's original 1887 article and also reviews the literature of possible Rothmund syndrome patients (Thannhauser, 1945). The patients described by Rothmund had similar overall appearance and their mental and physical development was normal. All of these patients had poikiloderma, and most of them had bilateral juvenile cataract. Later some of these patients were re-evaluated and only one of them was short, had small hands and feet and short extremities. Her hair started to grey and fall off and at the age of 40 she was bald (Thannhauser, 1945). Some decades later Thomson described three British patients who suffered from poikiloderma (Thomson, 1936). Two of the

patients were siblings so in their case a common genetic defect was the most likely explanation. However, there was a striking phenotypic difference between the siblings since the first one had only poikiloderma and a skeletal X-ray study revealed only slight disproportion in the skull bones giving her face a triangular appearance. The second sibling had poikiloderma accompanied by growth retardation and radial ray defect. She died at the age of one year because of an unknown cause. The third patient was a separated case with poikiloderma, but no striking bone anomalies. She had sparse hair, eyebrows and eyelashes and a facial resemblance with the first patient. None of these patients developed juvenile cataract. In both families the mode of inheritance was proposed to be autosomal recessive. Rothmund and Thomsons' syndromes were later combined into one entity and poikiloderma was chosen to be the hallmark feature (Taylor, 1957). Approximately 200 RTS cases are described in the literature (Vennos, et al., 1992).

Poikiloderma is a rash that is characterized by its acute and chronic phase (Vennos and James, 1995). In the beginning edematous plaques or erythematous patches appear on the cheeks and then start to spread to the extremities usually sparing the trunk. After the acute phase, the skin develops the typical poikilodermic appearance. It includes hypo- and hyperpigmented areas with atrophy and telangiectasia i.e. the widening of the capillaries (Thomson, 1936; Vennos, et al., 1992). Both sensitivity and insensitivity to the light has been reported and some patients have blistering of the skin during the acute phase. There are many syndromes which have poikiloderma like features or true poikiloderma as one of the symptoms (German and Takebe, 1989; Siegel, et al., 2003; Wang, et al., 2003a). Skin biopsies and histological examinations can be helpful to determine the type of dermatological changes. In RTS, poikiloderma usually appears at the age of three to six months and it has the typical pattern and distribution starting from the face and then spreading to the extremities (Vennos, et al., 1992). Later onset of poikiloderma at the age of one to three years is rare and these patients are considered to have atypical RTS (Wang, et al., 2003b).

As poikiloderma is the only constant finding in RTS patients large amount of additional symptoms have been reported in these patients. These findings include for example radial ray defects, growth retardation, cataract, alopecia, gastrointestinal findings and malignancies, mainly osteosarcomas (Moss, et al., 1990; Starr, et al., 1985; Wang, et al., 2001; Vennos, et al., 1992). In some patients chromosomal instability and defect in DNA repair has been found (Der Kaloustian, et al., 1990; Miozzo, et al., 1998; Orstavik, et al., 1994), but not in others (Dick, et al., 1982; Shuttleworth and Marks, 1987; Starr, et al., 1985). The differences in clinical findings suggest that RTS is a genetically heterogeneous syndrome.

2.4.1 Subgroup of Rothmund-Thomson syndrome with *RECQL4* mutations

Finding of mutations in the *RECQL4* gene from a subset of RTS patients proved the genetic heterogeneity in RTS (Kitao, et al., 1999b). Further mutation screenings among RTS patients have confirmed the initial finding (Balraj, et al., 2002; Beghini, et al., 2003; Broom, et al., 2006; Lindor, et al., 2000; Sznajer, et al., 2008; Wang, et al., 2003a; Wang, et al., 2003b; Wang, et al., 2002). In the most comprehensive study approximately 70% of the studied RTS patients had *RECQL4* mutations and most of them were truncating mutations (Wang, et al., 2003b).

Clinical data from the patients with *RECQL4* mutations is limited, but it gives clues which phenotypic features are most common among these patients. Usually patients have prenatal growth retardation, radial ray defects, alopecia, loss of eyebrows and eyelashes, gastrointestinal problems and osteosarcoma in addition to poikiloderma. None of the patients have developed bilateral juvenile cataract.

In this light it seems evident that patients described by Rothmund and Thomson very likely suffered from different clinical entities. Any patient with poikiloderma can potentially have RTS, but the major problem is that poikiloderma is not a specific feature. If the result of the differential diagnosis is RTS determining the subgroup might prove important because of the elevated osteosarcoma risk in RTS patients with *RECQL4* mutations. It has been suggested that Rothmund-Thomson syndrome should be separated into the RTSI and RTSII subtypes (Wang, et al., 2003b). Patients described by Rothmund (Thannhauser, 1945) represent the RTSI phenotype while patients with *RECQL4* mutations have the RTSII phenotype (Wang, et al., 2003b). According to Wang's classification a patient with atypical poikiloderma either in the form of distribution or the age of onset should have two additional features to substantiate the RTS diagnosis. These findings would be growth retardation, radial ray defects or skeletal dysplasia, osteosarcoma, cataract, alopecia or loss of eyebrows and eyelashes (Wang, et al., 2003b). It is peculiar that cataract is in the diagnostic criteria since bilateral juvenile it is not found in patients with *RECQL4* mutations (Balraj, et al., 2002; Beghini, et al., 2003; Broom, et al., 2006; Hilhorst-Hofstee, et al., 2000; Kant, et al., 1998; Kitao, et al., 1999b; Lindor, et al., 1996; Lindor, et al., 2000; Miozzo, et al., 1998; Pujol, et al., 2000; Sznajer, et al., 2008; Tong, 1995; Wang, et al., 2003b; Volpi, 2004).

RTSII phenotype is associated with elevated risk for osteosarcomas, but peculiarly not for other malignancies (Wang, et al., 2003b). Interestingly, *RECQL4* mutations do not seem to contribute to sporadic osteosarcoma (OS) cases (Nishijo, et al., 2004). RTSII patients develop osteosarcoma at a younger age than the patients in

sporadic cases, but their response to the treatment were not different from the sporadic cases (Hicks, et al., 2007). Only two RTS patient with the *RECQL4* mutations have developed another type of malignancy and both patients developed osteosarcoma first (Wang, et al., 2003b). Additionally, some patients have signs of the chromosomal instability, but these findings are not consistent (Broom, et al., 2006; Miozzo, et al., 1998).

Clinical data from the RTS patients with known *RECQL4* mutations and from two RTS cohorts are presented in Table 2. The first row contains data from 10 patients with the confirmed *RECQL4* mutations. A symptom was assumed to be absent if it was not given in the clinical data. This of course can lead to minor inaccuracies in the results as some of the features have been probably been unreported even if they were present. It is apparent that there is some overlap in described cases between these three groups (Table 2). For example some of the patients in the cohort of 41 patients likely belong to group of 10 RTS patients with *RECQL4* mutations. Additional features such as anal artresia, hearing loss, chromosomal instability, osteoporosis and problems with immunity are found occasionally. So far none of the patients has developed bilateral juvenile cataract. Some of the patients have irregularly shaped brownish spots and one of them has hyperpigmentation in the trunk which follows the lines of Blashchko (Broom, et al., 2006).

In the London medical database (www.lmdatabases.com/) craniosynostosis is listed as one of the features of RTS apparently based on the findings of Mégarbane and co-workers (Megarbane, et al., 2000). The patient was diagnosed with Baller-Gerold syndrome, but the clinical finding was also consistent with RTS. Craniosynostosis may have been unreported in some cases since Lisa Wang and Sharon Plon (personal communication) reported craniosynostosis in some cases of their RTS patients. Unfortunately, it is not known which of these patients had *RECQL4* mutations.

Table 2. Clinical data from three different sets of RTS patients.

	Poikiloderma	Short stature	Thumb anomaly	Radius anomaly	Patellar defect	Palatal defect	Osteosarcoma	GI problems	Alopecia/hair loss	Sparse brows and lashes
10 patients with <i>RECQL4</i> mutations	100	90	50	30	20	10	70	20	20	40
202 RTS cases from literature	100	70	27 [#]	-	-	-	*	-	80 ^{##}	
Cohort of 41 RTS patients	100	66	20 [#]		**	**	32	17	50	73

The values are given in percentages. In the first row the percentages are suggestive since the clinical data available was more thorough in some reports than in others. In the cohort of 41 patients the percentages are counted from the patients whose information was available; for example growth retardation was seen in 25 patients and this data was available from 38 of 41.

Thumb anomaly and radius anomaly combined. ## Alopecia/hairloss and sparse eyebrows and eyelashes combined. The authors state that the estimation of the frequency is difficult and that there were nine osteosarcoma cases in the literature (Wang, et al., 2001; Vennos and James, 1995). ** Found in some cases.*

Clinical and mutational data was collected from the following publications (Balraj, et al., 2002; Beghini, et al., 2003; Broom, et al., 2006; Kitao, et al., 1999b; Lindor, et al., 1996; Lindor, et al., 2000; Miozzo, et al., 1998; Pujol, et al., 2000; Sznajer, et al., 2008; Tong, 1995; Wang, et al., 2003b; Volpi, 2004). Data for 202 RTS cases collected from literature was taken from (Vennos and James, 1995) and the data of 41 RTS patients from (Wang, et al., 2001).

2.5 Baller-Gerold syndrome

The whole existence of Baller-Gerold syndrome (BGS, MIM 218600) as a separate entity has been widely debated (Cohen and Toriello, 1996; Huson, et al., 1990). The narrow definition of Baller-Gerold syndrome is craniosynostosis with radial ray defect, but there are patients with a more complex phenotype (Galea and Tolmie, 1990). Only about 20 cases have been described and there is a marked genetic heterogeneity because some of the patients first diagnosed as BGS have on further studies been found to have Fanconi anaemia, Roberts syndrome, VACTREL association or Saethre-Chotzen syndrome (Farrell, et al., 1994; Huson, et al., 1990; Rossbach, et al., 1996; Seto, et al., 2001). It has been also shown that valproate exposure during the fetal stage can lead to the BGS phenotype (de Oliveira, et al., 2005). The genetic heterogeneity and phenotypic overlap with other syndromes makes the BGS diagnosis complicated.

Subsets of patients with BGS have been found to have *RECQL4* mutations, but these patients have a broader phenotype than the narrow definition of BGS and phenotypic overlap with RTS was noted (Megarbane, et al., 2000). In addition to craniosynostosis and radial ray defect the patients suffered for example from pre- and postnatal growth retardation and poikiloderma (Megarbane, et al., 2000; Van Maldergem, et al., 1992).

2.6 *RECQL4* gene and protein

The *RECQL4* gene was cloned in 1998 and it was found to be a member of the *RecQ* gene family (Kitao, et al., 1998). *RECQL4* is located at the proximal end of the long arm of chromosome eight, 8q24.3 and its genomic size is about 6.4 kb and its coding sequence is 3.6 kb (Kitao, et al., 1999a). *RECQL4* is composed of 21 exons and 20 introns that are relatively small. In fact 13 out of the 20 introns are less than 100 bp long. Deletions in these small introns can lead to a missplicing of mRNA because the intronic size becomes too small for efficient splicing even if the required consensus sequences remain intact (Wang, et al., 2002). In *RECQL4* intronic deletions ranging from 11 to 25 bp have been reported in several cases (Balraj, et al., 2002; Broom, et al., 2006; Wang, et al., 2003b; Wang, et al., 2002). Schematic drawings of the *RECQL4* gene presented in Figure 2a.

RECQL4 has a wide expression pattern since it was present in all studied organs with the highest expression in thymus and testis (Kitao, et al., 1998). In synchronized cells the expression levels started to rise at the G1/S phase and were highest in the S phase of the cell cycle (Kitao, et al., 1998). In another study transformation of endothelial cells and fibroblasts with SV40*tsT* increased the

RECQL4 expression (Kawabe, et al., 2000). When B-lymphoplastoid cells were transfected with Epstein-Barr virus the expression of *RECQL4* started to rise when the cells proceeded to the S phase and the levels were highest in actively dividing cells in the S, G2 and M phases (Kawabe, et al., 2000). The phorbol myristic acetate (PMA) stimulation of these cells lead to the increase of *RECQL4* in later stages of the stimulation suggesting a role for it in DNA replication (Kawabe, et al., 2000).

RECQL4 encodes the RECQL4 protein of 1208 amino acids (Figure 2b). The localization of the RECQL4 protein has been shown to be nuclear in several studies (Burks, et al., 2007; Kitao, et al., 1998; Macris, et al., 2006; Woo, et al., 2006; Yin, et al., 2004). However in some reports RECQL4 has been found also in the cytoplasm (Burks, et al., 2007; Petkovic, et al., 2005; Yin, et al., 2004). It was suggested that the different antibodies and methods used in these studies could explain the inconsistencies (Burks, et al., 2007). The role of cytoplasmic RECQL4 is not known, but it was proposed to have a nuclear export signal that would allow it to shuttle between the nucleus and cytoplasm (Burks, et al., 2007). The analysis of mutant RECQL4 constructs has revealed nucleolar localization signal at amino acids 376-386 (Woo, et al., 2006). In another study the overlapping fragment at amino acids 364-492 was shown to contain nuclear localization signal in addition to nuclear retention signal (Burks, et al., 2007). The role of different cellular localization of RECQL4 protein is not yet understood.

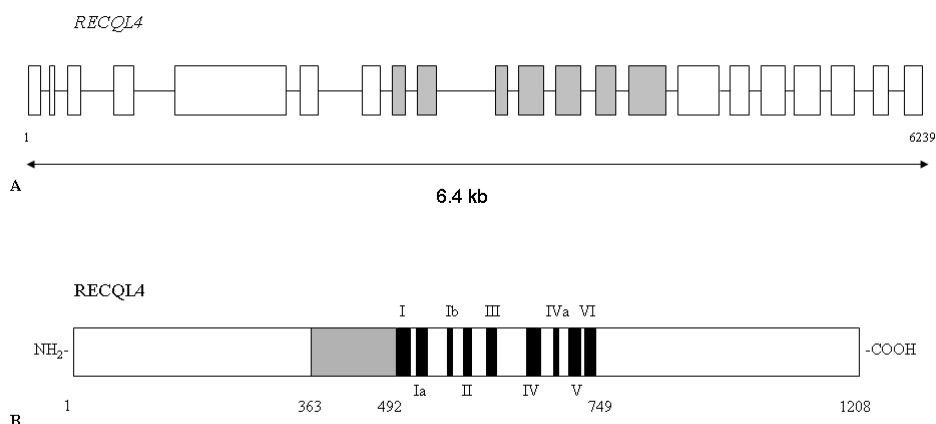


Figure 2. Schematic presentation of the *RECQL4* gene and *RECQL4* protein. A) The genomic structure of *RECQL4*. The exons 8-14 that contain the helicase domain are colored grey. B) The schematic structure of *RECQL4* protein. The conserved regions that are found in helicases are marked with black boxes. Nuclear localization signals found in *RECQL4* is marked with grey box.

2.6.1 *RecQ* gene family and helicases

RECQL4 was named after *E. coli* RecQ protein because of the protein sequence similarity (Kitao, et al., 1998). *E. coli* RecQ has a role in conjugal recombination and it has helicase activity (Nakayama, et al., 1984; Umezu, et al., 1990). In humans there are five RecQ orthologs that are *RECQL4* (Kitao, et al., 1998), *RECQL* (Puranam and Blackshear, 1994; Seki, et al., 1994), *BLM* (*RECQL3*, *RECQ2*) (Ellis, et al., 1995), *WRN* (*RECQL2*, *RECQ3*) (Yu, et al., 1996) and *RECQL5* (Kitao, et al., 1998) that share seven consensus domains common to the helicases (Kitao, et al., 1998). In addition, they share other conserved domains found in RecQ proteins such as the RQC (RecQ conserved region) and helicase-and-ribonuclease D/C-terminal (HRDC) domains (Kitao, et al., 1998). The HRDC domain that apparently is needed for the binding to the nucleic acid substrate (Morozov, et al., 1997). The schematic presentation of human RecQ proteins is given in Figure 3.

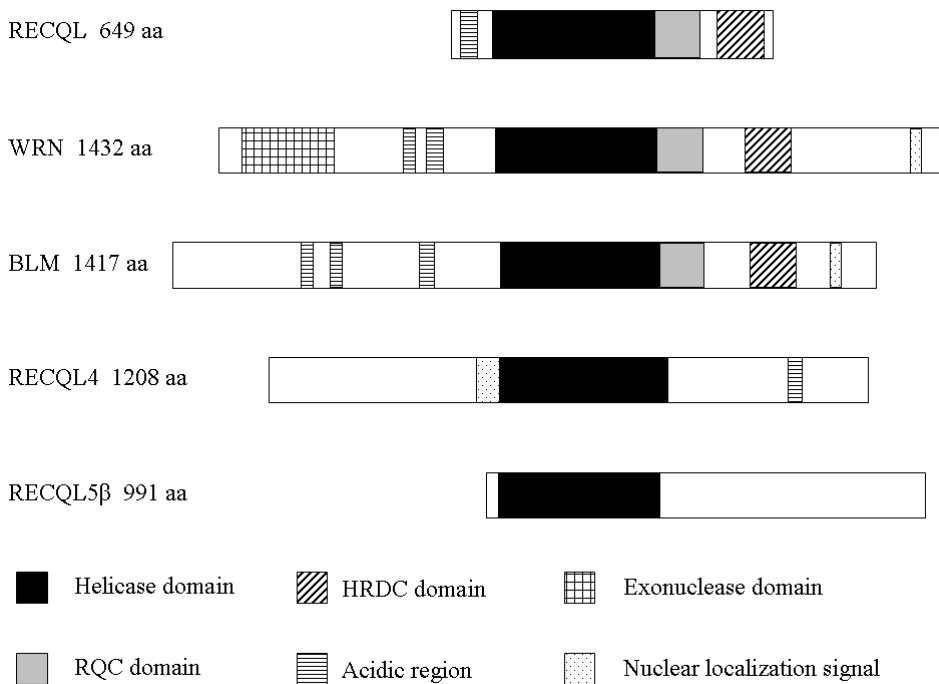


Figure 3. Schematic presentation of human *RecQ* proteins. The size of the polypeptide is given after its name. The conserved regions are marked in the diagram. Modified from (Harrigan and Bohr, 2003).

RECQL4 diverges most from the other long RECQ proteins since it lacks the HRDC domain present in other human RECQs and it has its acidic domain in its C-terminus whereas the other proteins have it in their N-terminal (Kitao, et al., 1998). Because of these differences it is proposed that RECQL4 might not have the same functions as the other RecQ proteins (Kitao, et al., 1998).

Helicase proteins are able to unwind either DNA or RNA substrate by using NTP hydrolysis as their energy source. They share characteristic conserved nine motifs and are classified into subgroups by the level of the sequence conservation. The role of different kind of helicases and their function has been widely studied, for a review see (Ellis, 1997; Gorbalenya and Koonin, 1993; Hall and Matson, 1999). The function of some of the areas is known. For example domains I and II are also known as Walker A and B domains and play roles in nucleotide binding (Walker, et al., 1982). Mutational analyses of different helicases in several species give clues to the importance of the specific conserved sequences, for a review see (Hall and Matson, 1999). Hall and Matson (1999) stated that all the motifs take part in the ATP binding and or hydrolysis at some level and they are needed for the function of the helicase.

All other human RecQ proteins - RECQL (Sharma, et al., 2005), BLM (Karow, et al., 1997), WRN (Gray, et al., 1997) and RECQL5 β (Garcia, et al., 2004) - have 3'->5' DNA helicase activity in contrast to RECQL4 (Macris, et al., 2006). In addition, WRN has exonuclease activity (Huang, et al., 1998). The RecQ proteins have several important roles such as DNA replication, recombination and telomere function in the maintenance of genomic integrity. Mutations in three of them *RECQL4*, *BLM* and *WRN* lead to recessively inherited syndromes.

2.6.1.1. Bloom and Werner syndromes

Mutations in *BLM* and *WRN* genes lead to Bloom (BLM, MIM 210900) (Ellis, et al., 1995) and Werner (WRN, MIM 277700) (Yu, et al., 1996) syndromes, respectively. Both are rare autosomally recessively inherited conditions with a high cancer predisposition and features of progeria.

Patients with Bloom syndrome are born small for their gestational age and a proportionate growth disturbance continues postnatally (Bloom, 1966). Patients are sensitive to sunlight and they show characteristic dermatological features such as telangiectasia and hypo- and hyperpigmentation. Patients have signs of the chromosomal instability such as spontaneous sister chromosome exchange possibly explaining their increased cancer risk. Males with Bloom syndrome are sterile and women have problems getting pregnant and their reproductive time is lower than unaffected women. Patients have increased risk to develop all kinds of malignancies.

An increased risk for colorectal cancer was reported among healthy BLM carriers, but the mechanism behind this phenomenon was not studied further (Gruber, et al., 2002). It was proposed that either the haploinsufficient cells are not able to maintain the genomic integrity or that a loss of the intact second *BLM* allele in a cell line would lead to a colony of BLM deficient cells (Gruber, et al., 2002).

Cells from BLM patients have a defect in the suppression of homologous recombination and thus a high rate of sister chromatid exchanges (SCE) in their chromosomes (Ellis, et al., 1995). BLM's role in the recombination is important since homologous recombination is needed during the double strand break (DSB) repair (Chen, et al., 2003; Wu, et al., 2001). BLM deficient cells also give a clue to their role in the DNA replication since the replication intermediates start to accumulate into these BLM deficient cells (Lonn, et al., 1990). Its expression profile which shows its highest expression in the S and G2 phases predicts this (Ababou, et al., 2000) and BLM associates with the late replication foci (Bischof, et al., 2001). It is also needed for the relocalization of the RAD50/MRE11/NBS1 complex into the sites of the arrested replication fork (Franchitto and Pichierri, 2002).

Werner syndrome is characterized by the growth disturbance due to the lack of growth spurt in their teens in addition to a stocky trunk and slender limbs (Thannhauser, 1945; Werner, 1904). Diabetes mellitus, scleroderma-like dermatological changes, cataracts, premature arteriosclerosis and subcutaneous calcification are common features. Patients have typical faces with beaked nose and their face is described prematurely aged with wrinkles and loss of subcutaneous fat. Patients are prone to develop sarcomas and they have marked chromosomal instability with deletions, translocations and inversions. Usually patients have several clonal cells in their blood or skin (Hoehn, et al., 1975). It has been shown that even though carriers of the *WRN* mutations are healthy they show signs of genetic instability with an increase risk for malignancies (Moser, et al., 2000).

WRN is needed in homologous recombination (Saintigny, et al., 2002) and in nonhomologous end-joining (Chen, et al., 2003). It is needed in the sites of the arrested replication fork to bypass the DNA damage by homologous recombination (Baynton, et al., 2003). WRN's role in solving the replication arrest was shown in studies with the hydroxyurea induced DNA damage (Constantinou, et al., 2000; Pichierri, et al., 2001). BLM and WRN have the ability to unwind the G4-DNA structures (Fry and Loeb, 1999; Sun, et al., 1998) which potentially give them the ability to unwind telomeric DNA.

2.6.2 Possible role and function of the *RECQL4* protein

As *RECQL4* belongs to the RecQ helicase family it was proposed to have a helical activity. Unfortunately, the extraction of the protein has been difficult and several groups have failed to extract it (Plon and Wang, 2006; Stagljar and Dietschy, 2005). Finally, the helicase activity test showed that *RECQL4* neither possesses helical nor translocase activities (Macris, et al., 2006; Yin, et al., 2004). The DNA dependent ATPase activity was shown in both studies (Macris, et al., 2006; Yin, et al., 2004). However, the exact role of the *RECQL4* protein is still unknown and difficulties with protein extraction are very likely to contribute to this situation.

Interactions of *RECQL4* with the UBR1 and UBR2 ligases have been demonstrated, but the meaning of these interactions is unknown (Yin, et al., 2004). *RECQL4* has been shown to partially colocalize with promyelotic leukaemia bodies (PML) and also partially colocalize with ssDNA regions after double strand breaks were introduced with etoposide (Petkovic, et al., 2005). Since *RECQL4* can form a complex with Rad51 it has been proposed that *RECQL4* has a role in the repair of the double strand breaks (DSB) lesions in cells (Petkovic, et al., 2005). Oxidative stress leads the *RECQL4* to localize in the nucleoli (Woo, et al., 2006). The C-terminal part of *RECQL4* was suggested to interact with poly(ADP-ribose) polymerase-1 (PARP-1). PARP-1 protein works in maintenance of genomic integrity (Grube and Burkle, 1992).

In *Xenopus* the *RECQL4* homolog is known as xRTS (Sangrithi, et al., 2005). This protein is needed for initiation of DNA replication and the human *RECQL4* can do this in cells depleted from xRTS (Sangrithi, et al., 2005). *RECQL4* seems to be important for the ability of murine cells to proliferate by allowing DNA replication (Ichikawa, et al., 2002; Sangrithi, et al., 2005). As the cells from human RTS patients or from the mice models are able to proliferate it was proposed that the N-terminus is the critical part for *RECQL4* function since none of the known patients with *RECQL4* mutations has truncating mutations in N-terminus of both of the alleles (Sangrithi, et al., 2005). In another study the N-terminus of *Xenopus* *RECQL4* homolog was responsible for the replication activity (Matsuno, et al., 2006). They showed that xRTS is required for polymerase alpha to bind chromatin and that it is needed for the replication machinery assembly in *Xenopus* (Matsuno, et al., 2006).

It is not known what happens to the mutated *RECQL4* polypeptide in patient cells. However, it has been shown that some of the *RECQL4* mutation bearing mRNAs become subject to nonsense mediated decay and a polypeptide is never produced (Beghini, et al., 2003; Sznajder, et al., 2008). In nonsense mediated mRNA decay premature stop codons are recognized and mRNA is degraded after its transcription (Losson and Lacroute, 1979). In Western blotting of proteins from RTS patient cells

RECQL4 was not detected (Broom, et al., 2006). This can be either due to a complete lack of the RECQL4 protein or that it is produced in such low amounts that it is not detected in Western blotting.

When cells from RTS patients with the *RECQL4* mutations were treated with H₂O₂ they were found to be hypersensitive for the stress and damage caused by oxidative species (Werner, et al., 2006). The cells had reduced growth because of proliferation failure due to a decreased DNA synthesis rate (Park, et al., 2006; Werner, et al., 2006). The UV-induced damage leads to the transient DNA synthesis inhibition in the RTS patient cells with *RECQL4* mutations (Park, et al., 2006). Wild type fibroblasts had a 24 hours arrest compared to eight hours in the RTS patient cells indicating that the cells would be defective in the S-phase arrest and that RECQL4 has role in the S-phase arrest (Park, et al., 2006). Since the cells from Bloom syndrome patients did not show this feature it is possible that RECQL4 has a unique function not seen in other RecQ proteins (Park, et al., 2006). Hydroxyurea (HU) causes the replication fork arrest in normal cells, but cells from RTS patient showed defect in S-phase arrest (Park, et al., 2006). Since the RECQL4 defective cells have problems either with the maintenance or the induction of the replication fork arrest RECQL4 could have a role in signalling of the DNA damage and/or repairing of it (Park, et al., 2006). The *RECQL4* transcription is shown to be downregulated by p53 in the G1 arrested cells (Sengupta, et al., 2005). One possible explanation for this phenomenon could be that the repression system might function as a back-up system in the surveillance of genomic stability (Sengupta, et al., 2005).

However, the exact role of human RECQL4 is still unknown. Circumstantial evidence shows that it could have role in the DNA repair and replication and in that way in the maintenance of the genomic integrity

2.6.3 *Recql4* deficient mouse models

Human RECQL4 and mouse Recql4 are 85.8% homologous in the protein level with three gaps in the alignment (Ohhata, et al., 2000). Two of them are 11 and 14 amino acid sequences that are missing from the mouse sequence. The largest gap size of a 35 amino acids is located in the area of human exon seven where human amino acid sequence has the gap (Ohhata, et al., 2000).

In total three different *Recql4* deficient mouse models have been generated. The first was generated by Ichikawa and co-workers and it had its exons five to eight replaced by the knockout vector (Hoki, et al., 2003; Ichikawa, et al., 2002). No viable knockout mice were produced since this disruption of the *Recql4* gene leads to the

death of embryos at 3.5-6.5 days after conception (Hoki, et al., 2003; Ichikawa, et al., 2002). Unfortunately, the original article is in Japan (Ichikawa, et al., 2002).

In the second mouse model exon 13 of the *Recql4* gene was deleted with an insertion of a targeting vector (Hoki, et al., 2003). In the mRNA level this leads to the inframe deletion of exon 13 and at the protein level it deletes 90 amino acids from the helical domain. In the mRNA level a substantial amount of truncated product containing exons 1 to 12 was found. The viability of these mice was extremely poor since only about 5% of the knockout mice survived more than two weeks after the birth. The embryogenesis of the $-/-$ mice was not disturbed and the lethality of the mice was postnatal. The mice were smaller than their littermates and developed hair loss and lesions on the skin. Hypoplasia was noted in several tissues such in bone, teeth, the villi of the small intestine and shrunken thymuses. Hoki and co-workers (2003) also state that the mice did not develop cataracts, as was in fact expected given the RTSII phenotype.

Interestingly in another study all the $-/-$ mice (Hoki, et al., 2003) died before embryonic day 10 (Yang, et al., 2006). Because of this the research group used $+/-$ mice that were heterozygotes for the mutant allele. Heterozygote mice were noted to have decreased bone mass. At the age of two months $+/-$ heterozygote mice had fewer osteoprogenitor cells in their bone marrow when compared to wild type mice. Recql4 protein was found in cells that were actively forming bone. For example in tibia the protein was abundant in chondrocytes of the growth plate, osteoblasts lining the trabecular surface and bone lining cells of endocortical surface. In osteocytes that are fully differentiated bone cells the protein was not detected. In the mouse osteoprogenitor cells MC3T3.E1 the overexpression of the Recql4 leads to the increased cell proliferation rate and the silencing of Recql4 by siRNA to a decrease in the cell proliferation (Yang, et al., 2006).

The third mouse model had a better survival rate since 84% of the knockout mice did survive to adulthood (Mann, et al., 2005). In this case the *Recql4* gene was disrupted by replacing the exon 9 to 13 by the PGKHprt mini gene that leads to the truncated mRNA containing exons 1 to 8. The phenotype of these mice was slightly different from the other mouse model. They did not have growth defect, but had more dramatic skeletal phenotype with palatal defects in all mouse studied and polydactyly in hind limbs in some of them. These mice had hypo-/hyperpigmentation in their tails. From 100 analyzed $-/-$ mice five developed cancers, three lymphomas and two osteosarcomas. Aneuploidy was noted in the mice and it was thought to be the major contributor to the cancers. The $-/-$ mice were crossed with $Apc^{Min/-}$ mice to generate a sensitized background in order to study the cancer susceptibility for gastrointestinal tumours. All the *Recql4* deficient $Recql4^{-/-}$, $Apc^{Min/-}$ mice developed intestinal tumours compared to 50% in $Recql4^{+/-}$, $Apc^{Min/-}$ (Mann, et al., 2005).

3 AIMS OF THE STUDY

Aims of this study were to:

1. Identify the gene and mutation(s) behind RAPADILINO syndrome.
2. Study the *RECQL4* gene from patients with suspected RAPADILINO, Rothmund-Thomson and Baller-Gerold syndromes.
3. Define the clinical phenotype of patients with *RECQL4* syndromes.
4. Make a genotype-phenotype correlation for *RECQL4* syndromes.

4 PATIENTS, MATERIALS AND METHODS

4.1 Patients and DNA samples

Originally, we had DNA samples from nine Finnish RAPADILINO families containing 11 patient samples for the initial genome-wide scan. In addition, we had 15 parental samples and 11 samples from unaffected siblings. When the mutations were found from the *RECQL4* gene we could screen additional RAPADILINO samples from patients or families. From one family we could not obtain the patient sample (r704), but we had parental samples, which were used to test the mutations. In family r1000 patient r1003 had a sibling who was deceased at the age of one year and she was suspected to have RAPADILINO syndrome. A paraffin embedded block was obtained for DNA extraction from patient r1203 and from the deceased sibling of patient r1003.

We obtained 274 Control DNA samples for estimation of the carrier frequency of the Fin_{major} mutation. In addition, we obtained 262 samples from patients who suffered from unexplained growth disturbance either in the form of pre- or postnatal growth retardation.

Clinicians outside Finland referred to us 33 index cases with two additional affected siblings in two families. In addition, we obtained 33 DNA samples from the parents and unaffected siblings of 15 families. We had no clear clinical data available about the patients and they were referred to us as having one of the *RECQL4* spectrum diagnoses. The clinical criteria used were very variable depending on the clinician's knowledge about *RECQL4* diseases. Some of the patients were claimed to have mild or atypical features. More thorough clinical data was requested from all the 18 non-Finnish patients with known *RECQL4* mutations and this was obtained in 17 cases (Publications II, III, IV).

The study was approved by the Ethical Committee of the Joint Authority for the Hospital District of Helsinki and Uusimaa.

4.2 Cell lines

Blood samples for lymphocyte extraction were requested from patients' r303, r304, r405, r504, r904 and r1003. Lymphocytes were collected from the fresh blood sample taken into a 10 ml heparin containing tube. In addition, we obtained skin biopsies from patients' r303, r304, r405, r605 and r1003 for fibroblast cell cultures.

4.3 Methods presented in articles

Materials and methods used in this thesis are presented in the original publications.

Method	Article
Minisequencing	I
Sequencing of the <i>RECQL4</i>	I, II, III, IV
<i>In situ</i> hybridisation	I
Cell cultures	I
mRNA studies	I
Genotyping	I
Collection of clinical data	IV
Bioinformatics	I, II, III, IV

4.4 Other methods

4.4.1 *Recombination and physical map of markers adjacent to RECQL4*

To determine the location of the markers adjacent to the *RECQL4* information of the markers D8S373, D8S1836 and D8S1925 with *RECQL4* was collected from the UCSC genome database (genome.ucsc.edu/) where the latest March 2006 assembly was used. To determine the recombination map of the given makers information was collected from Marshfield clinic's Centre for Human Genetics (research.marshfieldclinic.org/genetics/GeneticResearch/compMaps.asp).

4.4.2 *DNA extraction from paraffin embedded blocks*

DNA was extracted from two paraffin embedded tissue blocks by using a protocol modified from Fincka and co-workers' (Finke, et al., 1993). Five µm thick slices were cut from the paraffin block and about 30 of them were used in the DNA extraction. First paraffin was removed from the sample with Octane reagent (Flucka #74821). 1000 µl of Octane was added to the tube containing the paraffin slices and it was vortexed for 10 seconds. 100 µl of methanol was added and it was vortexed

again to dissolve the paraffin. The tube was centrifuged for two minutes at 13000 rpm (Eppendorf centrifuge 5810R, rotor F45-30-11). The supernatant was discarded and 360 µl proteinase K buffer (pH 8.0, 20 mg/ml proteinase K) was added and the contents of the tube was mixed by flicking. The solution was incubated in +55°C and vortexed three times over 48 hours.

After the incubation, the tubes were heated to +95°C for 20 minutes and centrifuged for 10 seconds with an Eppendorf centrifuge. A phenol-chloroform-isoamylalcohol mixture with the proportions 25:24:1, respectively was added to the tubes and they were vortexed for one minute. The tubes were then centrifuged for five minutes at 11000 rpm with an Eppendorf centrifuge at +4°C. The upper phase of the solution was then transferred to a new Eppendorf tube. 700 µl of cold (-20°C) 100% ethanol was added slowly to the tube. After this 400 µl of 0.2 M sodium acetate solution was added. The DNA was precipitated by flicking the tube and it was inverted 15 times to make sure the precipitation was complete. The solution containing the precipitated DNA was chilled for 30 minutes at -80°C and then centrifuged for 30 minutes in +4°C at a speed of 11000 rpm in an Eppendorf centrifuge. The supernatant was discarded and 1 ml of cold (-20°C) 70% ethanol was added to wash the DNA. The tube was then centrifuged as in the previous step. The washing of the DNA pellet was repeated with another 1 ml of ethanol and the centrifugation was 20 minutes in this second washing step. The supernatant was discarded and the DNA was air dried. The DNA was dissolved in 100 µl water and its concentration was measured using Nanodrop.

4.4.3 Cloning of the *RECQL4* constructs

The human and mouse *RECQL4* genes were cloned into the pEGFP and pCMV-Tag expression vectors. For the cloning of human full length wild type and mutant Δ ex7 *RECQL4* DNA fragments the pEGFP vector was cut open with *XhoI* and *Sall* restriction enzymes and pCMV-Tag with *EcoRI* and *Sall* restriction enzymes. For the cloning of mouse *Recql4* the pEGFP vector was cut with *EcoRI* and *ApaI* enzymes and pCMV-Tag with *EcoRI* and *Sall* enzymes. Enzymes were from Amersham Biosciences (*ApaI* # E1005Y, *EcoRI* #E1040Y, *Sall* # E1080Y and *XhoI* # E1094Y). In all cases either the GFP or FLAG-tag were incorporated into the N-terminus of the polypeptide. The cloned DNA fragment was amplified with primers containing a restriction site for enzymatic cleavage of the PCR product. Primer sequences are given in Table 3.

Table 3. Primers used for human and mouse *RECQL4* cloning.

Species	Enzyme	Primer sequence
Human 5'	<i>XhoI</i>	AATTCCTCGAGATGGAGCGGCTGCGGGACGTGCG
Human 5'	<i>EcoRI</i>	ATCTCGAATTCATGGAGCGGCTGCGGGACGTGCG
Human 3'	<i>Sall</i>	AATTCGTCGACTCAGCGGGCCACCTGCAGGA
Mouse 5'	<i>EcoRI</i>	ACTAGGAATTCATGGAGCGGCTGCGGACCGT
Mouse 3'	<i>Apal</i>	AATTCGGGGCCCTCATCGGCCTCTCAGCAGGA
Mouse 3'	<i>Sall</i>	AATTCGTCGACTCATCGGCCTCTCAGCAGGA

The PCR products and vectors were digested with corresponding restriction enzymes according to the manufacturers' instructions. Digested PCR product and vector were purified using QIAquick PCR Purification Kit (Qiagen #28104). The PCR products were ligated with their corresponding vectors with T4 DNA ligase (New England BioLabs #M0202S) according to manufacturers' instructions. The ligated product was then transformed into *E. coli* bacteria and the cells were plated on ampicillin containing culture dish. Bacteria clones were picked from the culture dish and grown in ampicillin containing media. Plasmid DNA was extracted according to manufacturer's protocol (Qiagen #12163).

4.4.4 Antibody production

Polypeptides of 15 amino acid from human and mouse *RECQL4* sequences were selected from antibody production in rabbits. Human polypeptide was EKRRWNEEPWESPAQ and mouse YYEEEEEEEEETMTD. Both of the polypeptides had MAP4 conjugates in their C-terminuses and they were produced by Invitrogen. In both cases 2680 µg of polypeptide was dissolved to prepare the antigen solution. Human polypeptide was dissolved into sterile water to obtain a 1670 µg/ml concentration. Mouse polypeptide was first dissolved into 20 µl of DMSO and then 1580 µl of sterile water was added. Two rabbits were immunized with each polypeptide so four rabbits were used in total. Before the first inoculation 0-blood samples from each rabbit was taken for a reference sample. The animals were inoculated four times with 400 µl of antigen solution in addition to 400 µl of adjuvant. Ten days after the fourth inoculation the animals were sacrificed and blood was drawn. The next day the blood was centrifuged and serum was stored at -20°C.

The number of National Public Health Institute permission for animal tests was 200132. Animal care and handling were at all times consistent with the guidelines set out in the National Research Council's guide for laboratory animal care and use.

4.4.5 Transfection of the cells

HeLa, COS-1 and HEK cells were transfected with pEGFP-Recql4, pEGFP-RECQL4 wild type, pEGFP-RECQL4 Δ ex7, pCMV-Tag-Recql4, pCMV-Tag-RECQL4 wild type and pCMV-Tag-RECQL4 Δ ex7 expression vectors. In addition, cells were transfected with empty pEGFP and pCMV-Tag vectors for the reference. The transfections were done according to the manufacturer's protocol by using 2 μ g of vector with 3 μ l of FuGENE HD (Roche, #04883560001). In double transfections with wild type and Δ ex7 mutant constructs 1 μ g of each construct was used. The cells were grown in 6-well plates with four glass slides on each well. After 24 hours post transfection the media was discarded and the wells were washed twice with PBS. The cells were fixed with 4% paraformaldehyde in PBS for 15 minutes and then the fixing media was replaced with PBS.

4.4.6 Immunostaining

The basic protocol for immunofluorescence staining was the following: The primary and secondary antibodies were different in various stainings. The PFA fixed cells on the glass slides were washed and incubated with PBS twice for 5 minutes. Cells were permeabilized with 0.1% Triton-X, 0.5% BSA in PBS for 15 minutes at room temperature. The cells were again washed twice for 5 minutes with PBS and blocking was performed with 0.5% BSA in PBS for 30 minutes. Primary antibodies were diluted into the blocking buffer at the following proportions 1:100 (rabbit 0-blood), 1:250 (rabbit antiserum for human and mouse polypeptides) and 1:500 (mouse anti-FLAG M2 antibody for pCMV-Tag vector, Stratagene #200472-21). Primary antibody was left on the cells for one hour after which the cells were washed three times with blocking buffer. Secondary antibodies were left on the cells for 40 minutes and the reaction was done in the dark. The secondary antibodies were diluted into the blocking buffer with a 1:200 concentration and the following antibodies were used: FITC conjugated Goat Anti-Rabbit IgG (Jackson Immuno Research laboratories #111-096-003), FITC-conjugated Goat Anti-Mouse IgG+IgM (Jackson Immuno Research laboratories #115-096-044), TRITC-conjugated Goat Anti-Mouse IgG (Jackson Immuno Research laboratories #115-026-003) and TexasRed dye conjugated Goat Anti-Rabbit IgG (Jackson Immuno Research laboratories #111-075-144). Because pEGFP expression vector has GFP attached to the expressed protein it does not need to be stained with antibodies. In double transfections and stainings where the pEGFP

expression vector was used red secondary antibody against primary anti-FLAG antibody was also used. In this way it was possible for us to distinguish the different antibodies from each other. After the secondary antibody the cells were washed twice for 5 minutes with PBS and once with milliQ water. Glass slides were mounted with Immu-Mount (Thermo #9990402).

4.4.7 Confocal microscopy

The cells were microscoped using a Leica DMRXE confocal microscope. Leica confocal software was used for taking of the pictures and the light intensity was kept the same while taking the pictures. The pictures were then processed with the CorelDRAW 12 program to add the scale bars and crop the pictures. Scale bars representing 20 μm were added to all pictures. Finally the pictures were opened in the Microsoft Office Picture Manager and the intensities of the pictures were autocorrected.

5 RESULTS AND DISCUSSION

5.1 Genotyping (I)

The whole genome scan was initially performed with 257 markers by using nine RAPADILINO families (see Section 4.1.), but no clear linkage peak was found. The scan marker D3S2398 in chromosome 3q28 gave LOD score 1.68, but the Transmission disequilibrium test - likelihood ratio test (TDT-LRT) value was 0.1065, which did not speak in favour of a true linkage. The observation by Dr. Robin Winter (Institute of Child Health, London, UK) that Rothmund-Thomson and RAPADILINO syndromes could be overlapping prompted us to study the 8q24.3 area which contained the *RECQL4* gene that was known to be mutated in Rothmund-Thomson syndrome (Kitao, et al., 1999b). The flanking markers D8S1836, D8S373 and D8S1925 were used to study whether Finnish RAPADILINO patients share a common haplotype in this area. The LOD scores for these markers were 1.98, 2.28 and 2.12, respectively with the corresponding TDT-LRT values 0.0628, 0.0033 and 0.0002. Surprisingly, several different haplotypes were observed in this 2.4 Mb area. Haplotype 18-7-6 was the major haplotype found in the families. Two completely different haplotypes were found from another chromosomes in families 3 and 4. The marker D8S272 was the last marker before telomere of the 8q chromosome in the original scan. This marker is only 6 Mb away from the marker D8S1836, but no evidence of linkage was found with this or any other markers further away.

The studied area of chromosome 8 is interesting since the physical order of the markers in the genome is - centromere-D8S1836-D8S373-D8S1925-telomere - but D8S373 and D8S1836 change places when the recombination map is studied. The area is 3.43 cM long when the average of both sexes is taken into account, but in females the distance between these markers is zero and in males 7.09 cM. This phenomenon of females having less recombinations in the telomeric end of the chromosome is present in all human chromosomes (Broman, et al., 1998). The lengths of male and female recombination maps differ from each other and in human the female map is 223.72 cM long and in males 113.05 cM when the sex average is 167.90 cM. The sizes in base pairs and cM are shown in more detail in Figure 4. There are two possible explanations for the very small shared haplotype. Either the mutation is very old or the haplotype became smaller when repeated recombinations degraded the ancestral chromosome or this chromosomal area is a recombination hot spot.

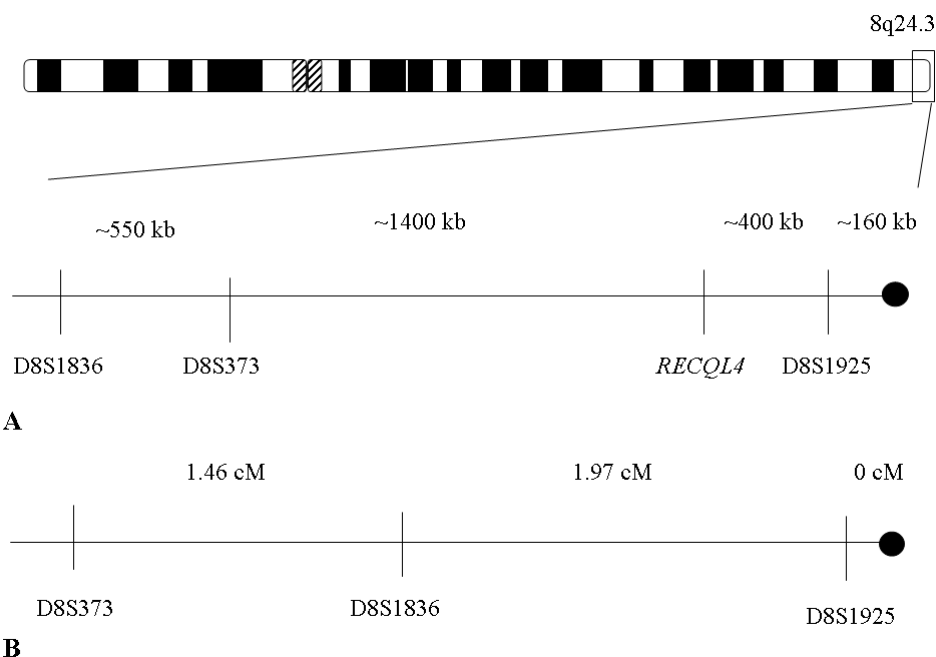


Figure 4. Schematic representation of chromosome 8 and the region containing the *RECQL4* gene and the markers D8S1836, D8S373 and D8S1925. The telomere of chromosome 8 is marked with a black dot. The distances between the markers and *RECQL4* are given in base pairs in the physical map (A) and in cM in the recombination map (B).

It is interesting that the common haplotype in RAPADILINO patients was so short and not constant in all studied families. This factor contributed to the fact that no linkage was found in the initial linkage analysis. The linkage could have been found if the second round of genome-wide scans had been performed since marker D8S373 was in that marker set. Even though the haplotype around the *RECQL4* gene was small, one could still assume that there was a common founder mutation behind the haplotype. In the Finnish disease heritage the founder mutations are commonly referred as Fin_{major} mutations. All the chromosomes shared the allele 6 of marker D8S1925. The haplotypes found in the patients and their parents are shown in Figure 5. Haplotype data from patient r903 was not available.

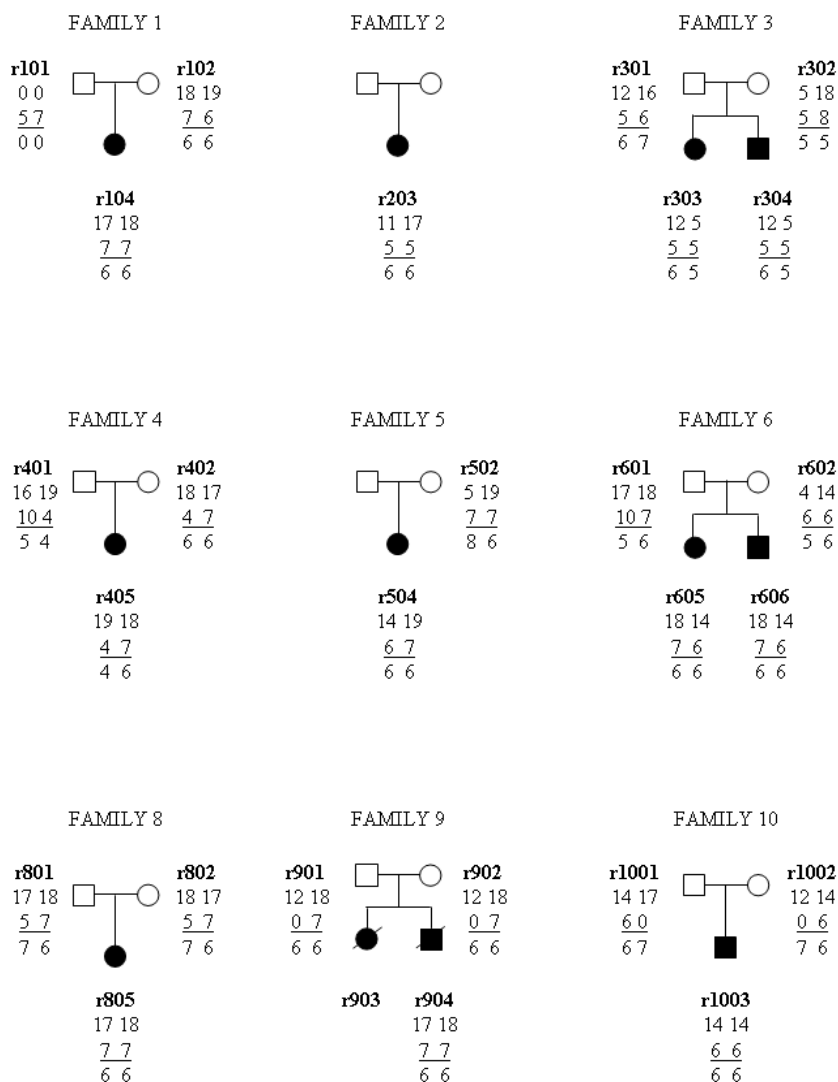


Figure 5. Observed haplotypes of the RAPADILINO patients and their parents. The haplotype contains data from three different markers and their order is D8S1836, D8S373 and D8S1925 from up to bottom. The RECQL4 gene is marked with a horizontal line between the markers D8S373 and D8S1925.

The birthplaces of the grandparents of the patients have two concentrations - one in the Savo region and another in Ostrobothnia. The birthplaces of grandparents in 11 families are shown in Figure 6. One has to bear in mind that in recessive inherited condition only half of the grandparents actually carry the mutation so the dots in Figure 6 do not actually present the mutations. Some concentration of the birthplaces of the patients' grandparents can be seen. Genealogical studies showed that patients in two families have a common ancestor when going back six generations. Their common ancestors were born in the beginning of the 19th century (Mentula, 2002). From the map it can be assumed that the Fin_{major} mutation could be new since most of the dots are found in the Savo area. In contrast the short haplotype speaks in favour of an old mutation, but the haplotype could have been also broken down just by chance e.g. if this chromosomal region is a recombination hot spot.



Figure 6. The birthplaces of the grandparents in 11 Finnish RAPADILINO families. The line represents the division between early and late settlement areas. The early settlement area is the coastal region of Finland and the late settlement area is the mainland. Modified from (Norio, 2003a).

5.2 Sequencing of the *RECQL4* gene (I, II, III, IV)

5.2.1 *RECQL4* mutations in Finnish RAPADILINO patients

In the sequencing analysis of the *RECQL4* gene we found altogether five different mutations in the Finnish RAPADILINO patients (Publications I and IV). The g.2545delT mutation i.e. the Fin_{major} mutation was found in all patient samples either in a homozygous or heterozygous form. In total 83% of the RAPADILINO chromosomes carried the Fin_{major} mutation that destroys the splice site of intron seven and results in an inframe skipping of exon seven. This was shown by analyzing cDNA that was reverse transcribed from the patients' mRNA. None of the healthy siblings or the parents had *RECQL4* mutations in both alleles.

Five compound heterozygote patients were found in four families. The second mutation was a unique truncating mutation in every family. In family 3 (patients r303 and r304), the second mutation was C>T transition that causes a premature stop codon p.Gln1091X (g.5721C>T). The mutation is located in exon 19. Another premature stop codon p.Arg1072X (g.5588A>T) was found in exon 18 in family 4 (patient r405). In family 7 no patient sample (r704) was available, but we found one of the parents to carry the g.2545delT mutation and another parent carried a novel premature stop codon p.Tpr269X (g.1556G>A) in exon five. The fourth truncating mutation was found in patient r1203 and it was a two nucleotide deletion in exon 21. This g.6295delCG mutation causes a frameshift. All compound heterozygote families had their own minor mutation and thus no other common ancestral mutation was found. The mutations found in the Finnish RAPADILINO patients are shown in Table 4 and in Figure 7 (Section 5.2.3.).

Table 4. Mutations found in the Finnish RAPADILINO patients.

Mutation	Outcome	Site	Found/Chromosomes
g.1556G>A	p.Tpr269X	Exon 5	1/30
g.2545delT	Skipping of exon 7	Intron 7	25/30
g.5588A>T	p.Arg1072X	Exon 18	1/30
g.5721C>T	p.Gln1091X	Exon 19	2/30
g.6295delCG	Frameshift	Exon 21	1/30

In family 10 two affected siblings were reported and the male patient r1003 was found to be homozygous for the Fin_{major} mutation. The phenotypes of the siblings have been previously reported (Similä, et al., 1983). Since the female sibling had died at the age of one year we obtained paraffin embedded tissue sample for the mutation analysis. We did not find any mutations and it seems that the female patient did not after all have the defect described in the report (unpublished data).

Two samples from the heterozygous patients were analysed to determine what was the fate of transcripts containing the g.5588A>T (p.Arg1072X) and g.5721C>T (p.Gln1091X) mutations since some of mRNAs containing premature stop codons are subject to a nonsense mediated mRNA decay. However, transcripts from both alleles were found in these two mRNA samples. Unfortunately, cell lines from patients r704 and r1203 were not available, but we had a cell line from the parental sample of r704 that was known to carry the g.1556G>A (p.Tpr269X) mutation. The cDNA fragment containing the g.1556G>A (p.Tpr269X) mutation was analyzed and heterozygosity for the mutation was found indicating that the mutation bearing allele is present in the mRNA level and not degraded in the cell.

5.2.2 Minisequencing of controls and patients with pre- or postnatal growth retardation

The estimation of the carrier frequency of the Fin_{major} mutation was analyzed from the sample of 274 healthy Finnish controls. Two heterozygotes were found and this gave a carrier frequency of one in 137 individuals. This is in concordance with the estimation of a new case approximately every second year. Yet this estimation is rough because of the small number of controls

In addition, 262 DNA samples of children with unspecified pre- and postnatal growth retardation were screened to study if there were mildly affected RAPADILINO patients among them. We found one homozygote for the Fin_{major} mutation (patient r1103 is patient 7 in Publication IV), but no heterozygotes at all (unpublished data). This highlights the problem of the carrier frequency estimation in cases where the allele frequency is low.

5.2.3 *RECQL4* mutations in Rothmund-Thomson, Baller-Gerold and non-Finnish RAPADILINO patients

All together 35 DNA samples taken from non-Finnish patients with *RECQL4* spectrum syndromes diagnosis were referred to us. *RECQL4* mutations were found in four RTS patients (Publications II and IV) and in five non-Finnish RAPADILINO patients (Publication IV). All these patients were index cases. Six BGS patients from four families were also found to have *RECQL4* mutations (Publication III and IV). Clinical data from one patient (patient 16 in Publication IV) was not available thus the diagnosis is left open. All together 15 novel *RECQL4* mutations were found in these patients (Publications II, III and IV, Figure 7). Two additional patient samples (patients 8 and 9 in Publication IV) were analyzed in another laboratory by Lisa Wang and Sharon Plon (mutation g.174del16 in Figure 8). Our laboratory has previously found two other novel mutations (g.3796T>C (p.Leu638Pro) and g.3506G>A (splice site)) (Sznajder, et al., 2008). The same p.Leu638Pro amino acid substitution was again found in a patient 16 in Publication IV.

Five of these novel mutations were amino acid substitutions g.5435C>T (p.Arg1021Trp) in Publication II and g.2633C>T (p.Pro466Leu), g.3797T>C (p.Phe637Ser), g.4076T>C (p.Phe697Leu), and g.5525A>G (p.Ile1051Val) in Publication IV. The remaining ten mutations were truncating: g.1246C>T (p.Gln166X), g.3768delCGGG (frameshift), g.3770delGGAG (frameshift), g.4043G>A (splice site), g.4473C>T (p.Gln800X), g.4410del22 (frameshift), g.4536C>T (p.Gln821X), g.4494insCGGGG (frameshift), g.5446delA (frameshift) (all in Publication IV) and g.5428A>C (splice site) (Publication III). Most of the *RECQL4* mutations are truncating and would thus lead to either loss of the protein or severely damage its function. The majority of the mutations have been found in the conserved helical region or in the latter third of the protein (Publication IV). Only a few mutations have been found from the first third of the *RECQL4* gene. Some truncating mutations lead to a nonsense mediated decay (Beghini, et al., 2003; Sznajder, et al., 2008) and in one report mutated *RECQL4* was not detected at the protein level (Broom, et al., 2006). Yet it is not known if the truncated *RECQL4* is present in low levels in the cells.

All the *RECQL4* mutations identified in Studies I, II, III and IV in addition to earlier published *RECQL4* mutations are presented in Figure 7.

5.2.4 The amino acid changes in the RECQL4 protein

Putatively pathogenic amino acid substitutions reported in Publications III and IV are presented in Table 5. The possible effects of the amino acid substitutions were tested using the PolyPhen and SIFT prediction programs as described in Study IV. In addition, 12 RECQL4 homolog sequences from different species were aligned to find most conserved amino acids as described in study IV. Some of the results are contradicting as for example p.Pro466Leu and p.Phe967Leu are predicted to be probably damaging by Polyphen as they change a rather conserved amino acid. Yet the SIFT predicts that these amino acid changes do not to affect protein function. The prediction of the amino acid change effect is difficult because of the lack of a 3D structure for RECQL4. Nor is there information about the interactions between RECQL4 and other proteins. In two cases p.Leu638Pro and p.Arg1021Trp the change to different type of amino acid could explain the deleterious effect to the protein. Proline is a ring structured amino acid and can potentially disrupt the polypeptide 3D structure. Tryptophan is the biggest amino acid and thus can disrupt the protein 3D structure.

Table 5. Putatively pathogenic amino acid substitutions in RECQL4 (Publications III and IV).

Mutation	Syndrome	PolyPhen		SIFT		Conservation
		Score	Pred.	Score	Pred.	
p.Pro466Leu	RAPADILINO	2.401	**	0.28	-	9/12
p.Phe637Ser	RAPADILINO	2.122	***	0.04	+	7/12
p.Leu638Pro	RTS	2.110	***	0.00	+	11/12
p.Phe697Leu	RAPADILINO	2.001	***	0.26	-	11/12
p.Arg1021Trp	RTS & BGS	0.558	*	0.01	+	4/9
p.Ile1051Val	BGS	0.119	*	0.70	-	6/11

*PolyPhen predictions are * Benign ** Possibly damaging *** Probably damaging. The cut off value for SIFT score is <0.05, + predicted to affect protein function, - predicted to be tolerated. Conservation is the number of same amino acids compared to all amino acids in a given certain position in the RECQL4 homolog alignment.*

In the alignment human RECQL4 protein was found to share similarities in the helical domain of RECQL4 of different species (data not shown). The conserved region in the helical domain is located between amino acids 492 to 749. In the alignment the human RECQL4 protein shared 51 identical and 31 similar amino acids in the region that included human RECQL4 sequence from amino acids 477 to 720. The homology between different proteins would have been higher if more distant sequences had been omitted from the alignment.

5.3 Phenotypes of RECQL4 syndrome patients and the genotype-phenotype correlation (I, II, III, IV)

Clinical data was collected from two Finnish (patients 6 and 7 in Table 6) and 13 non-Finnish patients with the diagnosis of RAPADILINO, RTS or BGS (Publications II, III and IV). In addition, medical records were collected also from the all other Finnish RAPADILINO patients in order to study their cancer status (Publication IV).

Table 6 shows the clinical findings in 15 patients with the *RECQL4* mutations. Patients 1-13 are in the same order as they appear in the original Publication IV (Table 3). Data concerning patients 14 and 15 in Study IV was omitted because of the large amount of the clinical data was not available as pregnancies were terminated. Patient 14 in Table 6 is the patient from the original Publication II. Patient 15 in this same table is the patient four of family 1 (Publication III). Clinical findings from three other affected siblings from this same family were left out because the first patient (patient 1, family 1) died immediately after the birth. Two subsequent pregnancies with affected foetuses were terminated. Patient 16 in Table 6 is the index patient of family 2 (Publication III). As seen in the table no patient has all the symptoms described.

Study of the clinical findings is important since the phenotype is complex. The lack of cataract in human RTS cases was considered peculiar. This lack of cataract was also mentioned in the report of the *Recql4* deficient mouse model (Hoki, et al., 2003). Our studies (Publications I, I, III, IV) show that cataracts are not present in RTS patients with the *RECQL4* mutations and thus should not be considered as an important diagnostic criterion.

Table 6. Clinical findings in 15 patients with *RECQL4* spectrum syndromes.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Diagnosis	A	A	A	A	A	A	A	B	B	B	B	B	C	B	C	C
Poikiloderma	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
Short stature >-2SD	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Craniosynostosis	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+
Brownish spots	-	-	+	+	-	+	+	-	-	-	+	-	-	-	-	-
Thumb a-/hypoplasia	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+
Radial a-/hypoplasia	+	+	+	+	+	+	-	+	+	+	+	-	+	+	-	+
Patellar a-/hypoplasia	+	+	+	+	-	+	-	+	+	+	+	-	-	+	+	+
Palatal defect	+	-	+	+	-	+	-	+	+	+	-	+	-	+	-	+
Osteopenia*	-	-	-	-	-	-	-	+	+	-	+	-	+	-	-	-
Malignancy	-	-	-	-	-	L	O	-	-	-	-	-	-	-	-	-
Diarrhoea	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-
Feeding problems	-	+	+	-	+	-	-	+	+	+	+	+	+	-	+	+
Hearing problems	+	-	-	-	-	-	-	+	+	-	-	-	+	-	-	-
Alopecia, hairloss	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	+
Joint dislocations	+	-	+	-	-	-	-	-	-	-	+	+	+	-	-	-
Gender	M	M	F	M	F	F	M	M	F	F	M	F	F	M	F	M
Mutations	g.2886delT, g.4076T>C	g.3797T>C, g.4628C>T	g.3768del4, g.4344C>T	g.4043G>A, g.5446delA	g.2633C>T, g.3770del4	g.2545delT, g.6295delCG	g.2545delT homozygote	g.2886delT, g.174del16	g.1798delAG, g.4344C>T	g.2886delT, g.4536C>T	g.2626G>A, g.4615G>C	g.1798delAG, g.4473C>T	g.4410del22 homozygote	g.2886delT, g.5435C>T	g.2886delT, g.5435C>T	g.5428A>C homozygote

Diagnosis; A RAPADILINO syndrome, B Rothmund-Thomson syndrome, C Baller-Gerold syndrome.

* Confirmed osteoporosis in patient 14. L Lymphoma, O osteosarcoma

RTS is phenotypically variable syndrome. Even when the patients with the *RECQL4* mutations are classified into their own subgroup a great clinical variability can be seen (Hilhorst-Hofstee, et al., 2000; Sznajer, et al., 2008). Some of the patients show very severe bone malformations when others have none or subclinical malformations. Based on clinical findings the distinction of patients who would be likely to carry *RECQL4* mutations is difficult because the symptoms are unspecific. In some cases *RECQL4* mutations are not found even though the phenotype and clinical findings suggest diagnoses of *RECQL4* syndromes.

BGS variability can be explained by the small number of patients and because the phenotype has similarities with other syndromes. It is still an open question whether BGS and RTS are in fact the same phenotype because craniosynostosis has been found in both of them. Patient 12 has the diagnosis of RTS even though craniosynostosis is present. Mutations found in RTS and BGS patients are mainly truncating mutations. The RTS patient in Publication II shares exactly the same combination of mutations as BGS patients from family 1 (Publication III). These patients were found to be compound heterozygotes for truncating g.2886delT mutation and for g.5435C>T transition that leads to the p.Arg1021Trp amino acid substitution. In family 1, the index patient died immediately after the birth and two following pregnancies with affected foetuses were terminated. Detailed clinical data is thus available only from patient 4. The major phenotypic difference between these patients is the presence of craniosynostosis in the BGS patients.

A total of 15 RAPADILINO patients are known in Finland. No other patients have been found even though a search was made of the birth malformation registry. We have noticed that like in RTS the RAPADILINO phenotype can have different levels of severity. The first patients assigned with RAPADILINO diagnosis were severely affected because the strict diagnostic criteria were used to find patients who would belong to same entity. Later more patients have come to our attention and two of them have a very mild phenotype. One patient with mild symptoms was diagnosed because he had an older affected sibling. Another RAPADILINO patient was found from a group of patients with pre- and/or postnatal growth retardation. He would not have been suspected to have RAPADILINO syndrome without the *RECQL4* mutation screening. The only dermatological finding in RAPADILINO patients is hyperpigmentation that resembles irregularly shaped café-au-lait spots. One mildly affected patient has plenty of these café-au-lait spots and they follow the patterning of the lines of Blaschko. These cells probably present a cell clone where the change leading to this hyperpigmentation occurred during embryogenesis and thus the lines of Blaschko have become visible. This same phenomenon is seen in a RTS patient (Broom, et al., 2006). RAPADILINO phenotype is characterised by growth retardation, radial ray and patellar defects, diarrhoea and feeding problems, joint

dislocations and high arched or cleft palate (Publications I and IV). The phenotype of Finnish RAPADILINO patients is summarized in Table 2 and in Publication I (Table 1). When the phenotypes of Finnish RAPADILINO patients (patients 6 and 7 in Table 6) is compared to the phenotypes of RTS and BGS patients in the same table the apparent differences are the lack of poikiloderma, craniosynostosis, alopecia, loss of eye brows and eye lashes in the RAPADILINO patients. Otherwise, the syndromes have very similar and overlapping clinical findings. This finding suggests the presence of modifying effects since the clinical findings in *RECQL4* syndrome patients are variable and some patients are very mildly affected.

5.4 Cancer predisposition in *RECQL4* syndromes

Cancer predisposition in *RECQL4* syndromes was initially thought to be restricted to osteosarcoma since in RTS this was the only malignancy reported in patients with *RECQL4* mutations and only isolated reports about other malignancies were reported in unspecified RTS patients. The osteosarcoma incidence in RTS varies between studies and when we combined published data with our clinical data (Publication II and IV) we found the osteosarcoma incidence to be about 43.6% among RTS patients (Publication IV). When the RAPADILINO and BGS patients were included the osteosarcoma incidence dropped to 30.6% (Publication IV). The reason why we found less osteosarcomas than previously reported is unknown. One explanation could be that patients in our studies (Publications I, II, III, IV) are still relatively young and they are still in the risk age.

Initially, we considered RAPADILINO patients to have lower malignancy risk than RTS patients (Publication I), but this hypothesis has been proven wrong. We found two osteosarcoma cases among Finnish RAPADILINO patients (Publications I and IV). Both of these patients are homozygotes for the g.2545delT mutation that leads to inframe skipping of exon 7. Thus there is significant osteosarcoma risk associated with all *RECQL4* mutations not just to the truncating forms (Wang, et al., 2003b). Interestingly, in addition to two osteosarcomas four (r704, r903, r904 and r1203) out of the 15 Finnish RAPADILINO patients have developed lymphoma (Publication IV). We showed that the Finnish RAPADILINO patients also have an increased lymphoma risk not seen in other patients with *RECQL4* mutations (Publication IV). The siblings r903 and r904 are both homozygotes for the g.2545delT mutation. Patients r704 and r1203 are compound heterozygotes for this mutation. The second mutation in patient r704 is g.1556G>A (p.Tpr269X) and in patient r1203 g.6295delCG mutation. It is possible that the lymphoma risk is due to the g.2545delT Fin_{major} mutation and the missing of exon seven could have critical role in hematopoietic cells. As the RAPADILINO phenotype is rare in other parts of the

world and some of the patients are still young the overall lymphoma risk for RAPADILINO or *RECQL4* syndrome patients is not yet known. However, the data collected from RTS patients does not show elevated lymphoma risk, but the reported clinical data has its flaws and it might not be complete.

Osteosarcomas and lymphomas were seen in one of the *Recql4* deficient mouse models (Mann, et al., 2005). In this model, the mutation generated truncated *Recql4* protein. It was encoded by first eight exons but lacked rest of the protein including the helicase domain that starts from exon 9. This type of truncating mutations are typically associated with osteosarcoma (Wang, et al., 2003b). However, this mouse model with the *Recql4* truncating mutation developed also lymphoma which has not been seen in humans with this type of mutations. Even though human and mouse cancers are not identical it is interesting that lymphomas are not seen in all the *RECQL4* spectrum syndromes. Time will show if the lymphoma risk stays associated only to the g.2545delT mutation leading to the skipping of exon 7.

A reason for malignancies in *RECQL4* syndromes is still unknown, but one explanation could be that *RECQL4* seems to function as a caretaker gene and if the *RECQL4* defect allows the cells to divide even when their genomic integrities have not been confirmed, aberrations in the genome could start to accumulate predisposing the *RECQL4* deficient patients to malignancies.

5.5 *In situ* hybridisation (I)

The expression levels of mouse *Recql4* were studied by using *in situ* hybridisation. The most prominent expression was detected in the chondrocytes of the developing bone and in the intestinal cell (see Figure 3 in Publication I). These findings fit nicely to the phenotypes of the *Recql4* deficient mouse models (Hoki, et al., 2003; Mann, et al., 2005).

Even though human and mouse protein functions may not be identical *in situ* hybridisation studies point to clinical findings seen in *RECQL4* spectrum syndrome patients. The most prominent are the bone malformations and the high expression of the *Recql4* in chondrocytes of the developing bone. Disturbed proliferation of the chondrocytes due to *RECQL4* deficiency can explain the bone malformations in humans. The intestinal problems seen in many patients are in concordance with the high expression in the mouse intestine.

5.6 Cell lines (I, unpublished)

When establishing Epstein-Barr virus (EBV) transformed cell lines from patient samples we found that the B-lymphocytes did not divide and grow at the same rate as control cells. Our first two attempts to grow EBV-transformed B-lymphocytes from patient r904 failed. After this the third sample from the same patient was left in to an incubator for weeks and we noticed some growth. This same procedure was followed in the treatments of five other samples (patients r303, r304, r405, r504 and r1003). In conclusion, the EBV-transformed B-lymphocyte cell line could be established if the cells were left to grow for weeks and eventually one could see typical cell clumps, but the growth rate of the culture remained slow. This phenomenon is apparently due to the *RECQL4* defect in the lymphoblastoid cells as cells from the heterozygote parents (samples r401, r402, r702) grew without problems. This problem could be specific to the Fin_{major} mutation since the lymphoblastoid cell line can be generated from the RTS patients without problems (Plon and Wang, 2006). We are aware of only one other case where lymphoblastoid cell line was not able to be produced (Miozzo, et al., 1998). This patient is homozygote for the g.2746del11 mutation. Apparently this problem is more common in Bloom syndrome where EBV-transformation of cells often fails (Kawabe, et al., 2000). Kawabe and co-workers (2000) stated that RecQ proteins are not essential for cell transformation (Kawabe, et al., 2000), but our findings with the observations from Bloom syndrome indicate that certain RecQ proteins might have role in it.

5.7 RECQL4 constructs and the results of transfections (unpublished)

Antibodies against human and mouse RECQL4 were tested in expression vector transfected cells with immunofluorescent staining. The rabbit antibody against mouse polypeptide gave staining with 0-blood that was supposed to be blank. Because of this the antibody was not used, as it was unspecific. Rabbit antibody against human RECQL4 was found to be specific when compared to 0-sample, but the amount of endogenous RECQL4 in HeLa cells was so low that it could not be detected reliably.

For localization studies of the human wild type and mutant (Δ ex7) RECQL4 HeLa, COS and HEK cells were transfected with GFP or CMV expression vectors containing either the wild type or mutant constructs. In total four different expression vectors were used. The transfection percentage was low with all the cell types and construct combinations. Usually the amount of transfected cells varied between 2 to 10%. In contrast for example when the cells were transfected with empty pEGFP vector all the cells were transfected and they expressed the

fluorescent GFP protein. In some transfected cells there was great overexpression of the RECQL4 protein that was seen as fluorescent aggregations in the cytoplasm. This phenomenon was especially strong in COS cells. The biggest problems were to find cells that were transfected but produced the protein in moderate levels to be able to see the assumed undisturbed localization of the protein. This generated another problem since taking pictures of the cells was somewhat difficult. The laser intensity was kept constant when taking the pictures and thus some of the moderately expressing cells were not properly visible against the black background. Because of this the pictures in Figure 8 were modified using Microsoft Office Picture Manager to autoformat the intensities. This of course changes the relative intensities of the pictures, but it was used to make the cells more visible.

In conclusion, wild type RECQL4 had mainly nuclear localization and Δ ex7 RECQL4 had mainly cytoplasmic localization in all the cells and constructs studied. This misslocalization of the mutant allele could explain the RAPADILINO phenotype. The mutated RECQL4 protein can get into the nucleus, but because of the lack of a nuclear retention signal it is not kept there. The results had some variation between different cell lines. In COS cells there were visible dots that were due to a high overexpression which lead to the protein accumulation in the cytoplasm. In HEK cells the staining was rather uniform between the nucleus and cytoplasm with a wild type construct. Our findings were in line with the findings of previously published studies (Burks, et al., 2007).

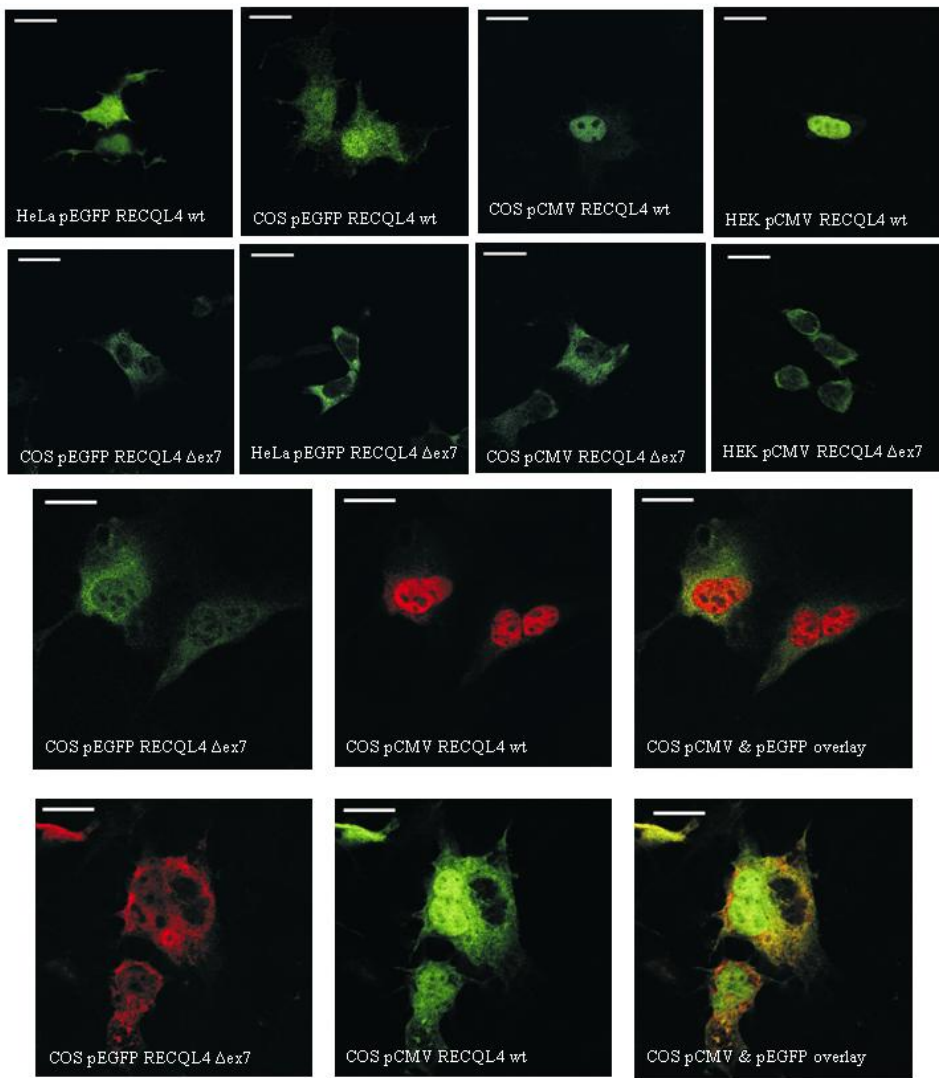


Figure 8. RECQL4 mutant and wild type expression in transfected HeLa, COS and HEK cells. The white scale bar in each picture is 20 μm.

6 CONCLUSION

Mutations in the *RECQL4* gene lead to RAPADILINO, Rothmund-Thomson and Baller-Gerold syndromes. Main common clinical findings are growth retardation, radial ray defect, gastrointestinal problems and elevated cancer risk, but poikiloderma and craniosynostosis separate them into distinct syndromes. RAPADILINO syndrome is a rare condition since only 20 RAPADILINO cases are known so far. However, RAPADILINO syndrome might be underdiagnosed since some of the patients have mild phenotypes that give no apparent clues to RAPADILINO.

Most of the 48 *RECQL4* mutations are truncating mutations, but the Finnish founder mutation is special since the mutation affects splice site and leads to inframe skipping of exon 7. The result of the mutation is a deletion of 44 amino acids from the *RECQL4* protein. This part of the *RECQL4* contains a nuclear retention signal and Δ ex7 mutant protein was found to have cytoplasmic localization whereas the wild type protein was located in the nucleus. All the Finnish RAPADILINO patients are either homozygotes or compound heterozygotes for this mutation.

RECQL4 mutations predispose patients to malignancies, which are osteosarcoma in RTS and osteosarcoma and lymphoma in RAPADILINO syndrome. Since all the patients with lymphoma are Finnish and carry the same mutation it is possible that the elevated lymphoma risk is due to this specific mutation (g.2545delT). Because the cancer risk is very high Finnish RAPADILINO patients need to be followed for possible lymphomas and osteosarcomas. This recommendation could be useful for all patients with *RECQL4* syndromes before more follow-up data accumulates.

It would be interesting to study if *RECQL4* mutations in healthy carriers would have phenotypic effect for example elevated cancer predisposition. Studies have shown that *RECQL4* mutations do not contribute to the sporadic osteosarcomas, but no studies concerning lymphoma patients have yet been done.

Even though these *RECQL4* syndromes are rare they give valuable information about the role of *RECQL4* in bone development and cancer. It is interesting how different mutations in *RECQL4* can lead to different phenotypes, but our understanding of this genotype-phenotype correlation is still in its early stages.

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